Journal of Cell Science 112, 2381-2390 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
JCS0273

Functionally homologous DNA replication genes in fission and budding yeast

Mar Sánchez, Arturo Calzada and Avelino Bueno*

Instituto de Microbiología-Bioquímica/Centro de Investigación del Cáncer, Departamento de Microbiología y Genética, Edificio Departamental, Campus Miguel de Unamuno, CSIC/Universidad de Salamanca, 37007 Salamanca, Spain
*Author for correspondence (e-mail: abn@gugu.usal.es)

Accepted 3 May; published on WWW 24 June 1999

SUMMARY

The *cdc18*$^+$ gene of the fission yeast *Schizosaccharomyces pombe* is involved in the initiation of DNA replication as well as in coupling the S phase to mitosis. In this work, we show that the *Saccharomyces cerevisiae CDC6* gene complements *cdc18-K46* ts and *cdc18* deletion mutant *S. pombe* strains. The budding yeast gene suppresses both the initiation and the checkpoint defects associated with the lack of *cdc18*$^+$. The Cdc6 protein interacts in vivo with Cdc2 kinase complexes. Interestingly, Cdc6 is an in vitro substrate for Cdc13/Cdc2 and Cig1/Cdc2, but not for Cig2/Cdc2-associated kinases. Overexpression of Cdc6 in fission yeast induces multiple rounds of S-phase in the absence of mitosis and cell division. This *CDC6*-dependent continuous DNA synthesis phenotype is independent of the presence of a functional *cdc18*$^+$ gene product and, significantly, requires only Cig2/Cdc2-associated kinase activity. Finally, these *S. pombe* over-replicating cells do not require any protein synthesis other than that of Cdc6. Our data strongly suggest that *CDC6* and *cdc18*$^+$ are functional homologues and also support the idea that controls restricting genome duplication diverge in fission and budding yeast.

Key words: *CDC6* /*cdc18*$^+$, Cell cycle, Replication control, Fission yeast, Budding yeast

INTRODUCTION

All eukaryotes share the cell division cycle problems derived from proper alternation of chromosomal DNA duplication during the S phase, with segregation of chromosomes at mitosis that guarantees a single genome replication during each cell division. These two distinct processes must be interdependent to the extent that no cell ever undergoes an extra round of DNA replication without an intervening mitosis and that nuclear division never occurs before replication has been completed. In order to ensure this interdependency, eukaryotes must have a control mechanism that prevents an increase in ploidy or missegregation of unreplicated chromosomes from taking place. Given the common nature of the problems that cells face during each cell division, proteins participating in the initiation of chromosomal DNA replication and proteins responsible for the coupling of replication and mitosis are likely to be widely conserved in eukaryotes.

Among the cell division cycle temperature-sensitive mutants isolated in yeast that are unable to initiate the S phase at the restrictive temperature, budding yeast *CDC6* has the properties of a central regulator in the initiation of DNA replication in eukaryotes. Early work on *cdc6-1* ts mutants suggested that the Cdc6 protein acts at the G_1-to-S phase transition (Hartwell, 1976). It has been suggested that the Cdc6-1 protein is defective in its interaction with yeast autonomously replicating sequences (ARS). ARSs are known to be DNA replication origins (Hogan and Koshland, 1992). In fact, origin firing is defective in *cdc6-1* mutant cells (Liang et al., 1995). In *S. cerevisiae*, origin activation relies on the formation at ARSs of the pre-replicative complex, a multi protein subunit formed by at least the six proteins of the origin recognition complex (ORC), Mcms, Cdc45, Cdc6, Cdc7 and Dbf4 (Bell and Stillman, 1992; Diffley and Cocker, 1992; Diffley et al., 1994; Dowell et al., 1994; Liang et al., 1995; Zou et al., 1997; Tanaka et al., 1997; Aparicio et al., 1997; Donovan et al., 1997). It has been shown that formation of the pre-replicative complex is dependent upon the presence of a functional *CDC6* gene (Cocker et al., 1996). A *Xenopus* homologue of Cdc6p has been cloned and molecularly characterized (Coleman et al., 1996). XCdc6 is essential for the initiation of DNA replication in frog cell-free extracts and its physical interaction with chromatin is needed in order to allow XMcm binding to chromatin. Recently, a human homologue of Cdc6p/Cdc18p initiator proteins, named hCDC6, has also been isolated (Williams et al., 1997; Hateboer et al., 1998). Likewise the *Xenopus* homologue, hCDC6 is esssential for the initiation of DNA replication in human cells cultivated in vitro (Hateboer et al., 1998; Yan et al., 1998). In conclusion, the identification of a family of proteins performing a similar and crucial role in the initiation of DNA replication in yeast and animal cells supports the idea that the mechanism underlying the control of genome duplication in eukaryotes would be highly conserved.

It is remarkable that Cdc6/Cdc18 defective cells do undergo

cell division even in the absence of DNA replication (Kelly et al., 1993; Piatti et al., 1995). In *S. cerevisiae*, Cdc6 protein levels oscillate during the cell cycle (Piatti et al., 1995). Consistent with a role in preventing mitosis, constitutive expression of Cdc6 in budding yeast delays cell cycle progression in G_2 (Bueno and Russell, 1992; Basco et al., 1995; Elsasser et al., 1996), as is the case for Cdc18 in *S. pombe* (Kelly et al., 1993; Muzi-Falconi, 1996; Nishitani and Nurse, 1995). Furthermore, Cdc6 rescues mitotic catastrophe in *S. pombe cdc2-3w wee1-50* and reverses lethality of op-*cdc25* and *cdc2-3w* fission yeast strains in the presence of hydroxyurea (Bueno and Russell, 1992), indicating that the *S. cerevisiae CDC6* gene can function as a potent M phase inhibitor both in budding and fission yeast (Bueno and Russell, 1992). These data suggest that the pre-replicative complex may provide a signal that prevents mitosis until DNA replication has been initiated. We have been interested in determining whether Cdc18 and Cdc6 define a class of proteins with dual functions in yeast. Here we report that Cdc6 substitutes Cdc18 in *S. pombe* strains deficient for the initiator protein. *CDC6* complements both the initiation and the coupling of the S phase to mitosis defects associated with the lack of *cdc18+*. Our data indicate that Cdc6 and Cdc18 are homologous.

MATERIALS AND METHODS

Strains and plasmids

All *S. pombe* strains were derived from the wild-type 972h$^-$. All strains used in this study were *ade6*-704, *leu1*-32 and *ura4*-d18 except where otherwise specified. The temperature-sensitive and deletion mutants used were: *nmt1:CDC6HA(sup3-5); nmt1:CDC6HA(sup3-5) pDW232ntf1*$^+$*; cdc18-K46; cdc18-K46 nmt1:CDC6HA(sup3-5); cdc18-K46 nmt1:CDC6HA(sup3-5) pDW232ntf1*$^+$*; cdc18::ura4*$^+$ *nmt1:CDC6HA(sup3-5); cdc2-33; cdc2-33 nmt1:CDC6HA(sup3-5); cig1::ura4*$^+$ *nmt1:CDC6HA(sup3-5) pIRT2ntf1*$^+$*; cig2::LEU2 nmt1:CDC6HA(sup3-5) pDW232ntf1*$^+$*; cig1::ura4*$^+$ *nmt1:CDC6*^{264}*HA(sup3-5); cig2::ura4*$^+$ *nmt1:CDC6*^{264}*HA(sup3-5); nmt1:cdc18*$^+$ *(sup3-5); and cig2::LEU2 nmt1:cdc18*$^+$ *(sup3-5).*

To construct the plasmid *pREP5CDC6HA6His*, the *Nde*I-*Not*I *CDC6* fragment amplified by PCR (Sadhu et al., 1990) was inserted into the*pREP5cdc2HA6His* vector (Millar et al., 1991) cut with *Nde*I-*Not*I to remove the *cdc2*$^+$ gene. The 33mer 5′ oligonucleotide used for this PCR had the sequence: 5′-CGGATCCATATGTCACTATACC-AATAACTCCA-3′, where the underlined part of the sequence contains *Bam*HI and *Nde*I sites and the 3′proximal 24 nucleotides correspond to the beginning of the *CDC6* ORF.

The 28mer 3′oligonucleotide had the sequence: 5′-TGCGGCCGCGGTGAAGGAAAGGTTTCAA-3′, the underlined sequence containing a *Not*I site and the reverse complement of the 3′proximal 19 nucleotides corresponding to the 3′end of the *CDC6* ORF, without the stop codon. The PCR reaction products were digested with *Nde*I-*Not*I and separated by agarose gel (1%) electrophoresis. The resulting 1.5 kb purified band was cloned as described (Sambrook et al., 1989). A similar strategy was used to construct the plasmid pREP5*cdc18*$^+$. The oligonucleotides used had the sequence: 5′-CCATATGTGTGAAACTCCA-3′, where the underlined part of the sequence contains a *Nde*I site and the 3′ proximal 15 nucleotides correspond to the beginning of the *cdc18*$^+$ ORF, and 5′-TGCGGCCGCGTCTTCTGTCAAAAAATCG-3′, the underlined sequence containing a *Not*I site and the reverse complement of the 3′proximal 18 nucleotides corresponding to the 3′end of the *cdc18*$^+$ ORF, without the stop codon.

Integrative transformations were carried out as described (Moreno et al., 1991). Selected single integrants were maintained on MM plates plus thiamine, and expression from the *nmt1*$^+$ promoter was induced after washing three times in MM or by streaking onto MM plates.

Gene disruption of *cdc18*$^+$

Flanking regions of the *cdc18*$^+$ gene were amplified by PCR and cloned into pGEM-T to form p5′cdc18 and p3′cdc18 plasmids. The sequences of the four oligos used for this purpose were: (oligo I) 5′-ATGGATCCAGGTTCGCGCGTAGAACG-3′, (oligo II) 5′-AT-GCGGCCGCAAGCTTTTTGTGGGTGTTTGGAAATGT-3′, (oligo III) 5′-ATGCGGCCGCAAGCTTGTGTCTTCGAGATCGTTTGAT-3′, (oligo IV) 5′-ATAGATCTAACTTAGATTAAAGTGTT-3′.

Using pairs I and II, a left 441 bp PCR fragment of the flanking genomic sequence of *cdc18*$^+$ was generated, flanked by *Bam*HI and *Hin*dIII-*Not*I (underlined sequences). With pairs III and IV, a right 450 bp PCR fragment of the flanking genomic sequence of *cdc18*$^+$ flanked by *Not*I-*Hin*dIII and *Bgl*II (underlined sequences) was obtained. PCR fragments were cloned into the pGEMT vector to generate p5′cdc18 and p3′cdc18. p5′cdc18 was cut with *Not*I, treated with alkaline phosphatase, electrophoresed and gene-cleaned. p3′cdc18 was also cut with *Not*I and the 0.4 kb insert purified after electrophoresis. This 0.4 kb fragment was inserted into the p5′cdc18 *Not*I fragment to form p5′3′cdc18. p5′3′cdc18 was cut with *Hin*dIII, treated with alkaline phosphatase, electrophoresed and gene-cleaned. A *Hin*dIII 1.8 kb DNA fragment containing the *ura4+* gene was inserted between the flanking PCR fragments to form pGEMT*cdc18*::*ura4+*. This plasmid was used to delete wild-type *cdc18*$^+$ in a *leu1*-32,*ura4*-d18, *ade6*-704, *nmt1:CDC6HA(sup3-5)*, as described in results.

Culture conditions and general techniques

All media used to culture *S. pombe* strains have been described by Moreno et al. (1991). All reagents were supplied by Sigma or Fischer Scientific. General molecular techniques were performed as described by Sambrook et al. (1989) and Moreno et al. (1991).

Cells were cultured in YES (Moreno et al., 1991) except when selecting for plasmids. In the latter case, cultures were grown in minimal medium (MM) with supplemented amino acids. General molecular techniques were performed as described (Sambrook et al., 1989; Guthrie and Fink, 1991). Temperature-sensitive mutants were cultured at 25°C, the permissive temperature, or at 35.5°C, i.e. the restrictive temperature. Wild-type cells were cultured at 32°C or as indicated.

Thiamine and cycloheximide were used at final concentrations of 5 μg/ml and 25 μg/ml, respectively.

Nuclear staining and flow cytometry

Cells were fixed and stained with 4′,6-diamino-2-phenylindole (DAPI), as described (Moreno et al., 1991). Stained cells were visualised using a fluorescence microscope (Zeiss).

The DNA content of individual cells was measured using a Becton Dickinson FACScan apparatus. Cells were fixed and processed for flow cytometry by the method of Hutter and Eipel (1979), staining them with propidium iodide.

Production of Cdc6Ha protein in *E. coli*

The *Nde*I-*Bam*HI *CDC6HA* fragment was cloned into the bacterial expression vector pT7-7. *E. coli* BL21-DE3 cells transformed with the resulting plasmid were induced to express the fusion protein by adding IPTG. The Cdc6Ha fusion protein was purified to homogeneity from inclusion bodies by solubilization in 6 mM NaH_2PO_4, 4 mM Na_2HPO_4, 2% SDS pH 6.8 buffer. Solubilised protein was loaded into an 11% SDS-PAGE gel and purified by electroelution. SDS was removed and the fusion protein was refolded by dialysing it against 25 mM Tris-HCl, 192 mM glycine, pH 8.3, buffer. Protein concentrations were estimated by comparison with BSA standards.

Protein extract preparation

Soluble protein extracts were prepared as previously described (Moreno et al., 1991). About 2×10^8 cells were collected, washed and

broken with glass beads in HB buffer. The HB buffer contained 60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 25 mM MOPS (pH 7.2), 15 mM $MgCl_2$, 15 mM EGTA, 1 mM DTT, 0.1 mM sodium orthovanadate, 1 mM PMSF, and 20 μg/ml of leupeptine and aprotinin. The glass beads were washed with 500 μl of HB buffer and the total extract was recovered and centrifuged using an Eppendorf microfuge at 15,000 rpm for 15 minutes at 4°C. The supernatant was used and protein concentration was measured using the BCA assay kit (Pierce).

Western blotting and immunodetection of Cdc6 protein

Protein extracts and immunoprecipitates were electrophoresed using 10% SDS-polyacrylamide gels (Laemmli, 1970). For western blots, 40 μg of total protein extracts from each sample were blotted to nitrocellulose. The antibody used to immunodetect Cdc6 was 12CA5 anti-Ha monoclonal antibody (Boehringer Mannheim) (1:500). Proteins were detected using horseradish peroxidase-conjugated anti-mouse antibody and an ECL kit (Amersham, Bucks, UK).

Kinase activity assays

$p34^{cdc2}$ protein kinase activity was immunoprecipitated from 1 mg of soluble extracts using 2 μl of C2 anti-Cdc2, 2 μl of SP4 anti-Cdc13, 2 μl of 9830-U anti-Cig1 (Mondesert et al., 1996), or 2 μl of anti-Cig2 (Martín-Castellanos et al., 1996) antibodies for 1 hour and immunocomplexes were then collected with 30 μl of Protein A-Sepharose. Immunoprecipitates were washed six times with HB buffer and incubated in 50 μl of kinase buffer (HB buffer containing 0.1 mM ATP, 20 μCi/ml [γ-^{32}P]ATP and 1 mg/ml of the appropriate substrate, either histone H1 or Cdc6Ha-purified protein). Reactions were incubated at 30°C for 30 minutes, stopped with SDS sample buffer, and denatured for 5 minutes at 100°C. Samples were run on a 12% SDS-polyacrylamide gel. Phosphorylated histone H1 was detected by autoradiography and quantitated using a Fujifilm BAS 1200 PhosphorImager.

RESULTS

The *S. cerevisiae CDC6* gene is a functional homologue of *S. pombe cdc18*$^+$

It has previously been shown that Cdc6 and Cdc18 share 28% identity along their predicted primary protein sequence (Kelly et al., 1993). Both proteins perform comparable functions in budding and fission yeast, respectively (Piatti et al., 1995; Kelly et al., 1993). Therefore, to investigate whether *CDC6* and *cdc18*$^+$ perform homologous cell cycle control functions in the two eukaryotic organisms, we constructed *S. pombe cdc18-K46 nmt1:CDC6HA* (*sup3-5*) strains in an attemp to determine whether the expression of Cdc6 rescues the temperature-sensitive defect associated with the *cdc18-K46* allele. This single mutant arrests at 35.5°C with an elongated phenotype and a 2C DNA content (Nasmyth and Nurse, 1981; Kelly et al., 1993). All *cdc18-K46 nmt1:CDC6HA* cells were able to form colonies capable of supporting growth at 35.5°C (Fig. 1). Rescue was not observed when the medium contained thiamine, which represses the *nmt1* promoter. This test shows that *CDC6* expression suppresses the ts defect of a *cdc18-K46* fission yeast strain.

While the fission yeast deleted for *cdc18*$^+$ shows a dual defect, not initiating DNA replication and actively dividing (Kelly et al., 1993), *cdc18-K46 S. pombe* strains seem to be only defective for DNA replication, arresting without progressing into cell division (Nasmyth and Nurse, 1981). These observations suggest the maintenance of the checkpoint coupling initiation of the S-phase to mitosis in the latter. Accordingly, we checked whether *nmt1:CDC6HA* was able to complement the deletion of *cdc18*$^+$. The 2.6 *Sac*I-*Sph*I restriction fragment from pGEMT*cdc18*::*ura4*$^+$, containing the *ura4*$^+$ marker flanked by *cdc18*$^+$ genomic sequences, was used to transform a *nmt1:CDC6HA(sup3-5) S. pombe* strain. Transformants were selected in minimal medium lacking both uracil and thiamine and tested by Southern blot for the correct replacement of the *cdc18*$^+$ wild-type gene with the *cdc18*::*ura4*$^+$ (Fig. 2A,B). 9%

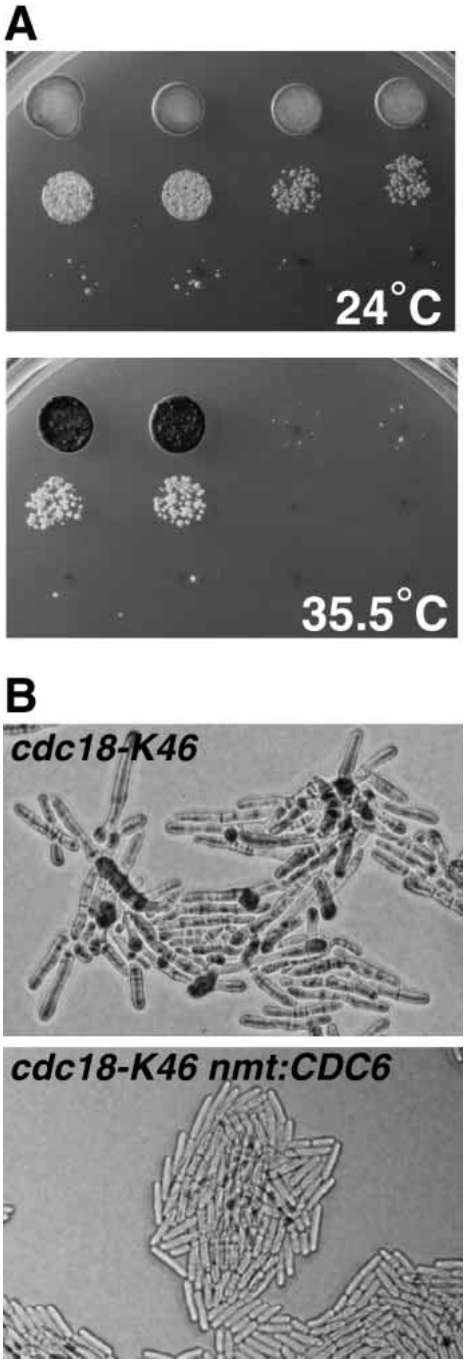

Fig. 1. Suppression of *cdc18-K46* by expression of *CDC6* in *S. pombe*. (A) Different dilutions of duplicate samples of *cdc18-K46* (two on the right) and *cdc18-K46 nmt1:CDC6 (sup3-5)* (two on the left) strains growing on plates of minimal medium at 24°C or 35.5°C, as indicated. (B) *cdc18-K46* or *cdc18-K46 nmt1:CDC6 (sup3-5)* cells photographed from a Petri dish incubated at 35.5°C.

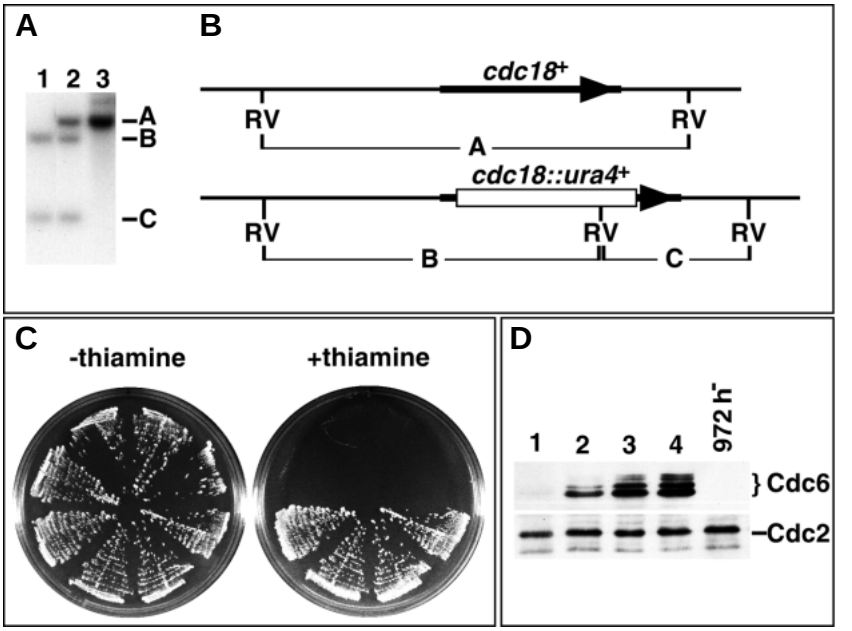

Fig. 2. Overexpression of *CDC6* rescues the deletion of *cdc18* in the fission yeast. (A) Deletion of *cdc18*$^+$ in a *nmt1:CDC6* integrant. Southern hybridization of a haploid strain deleted for *cdc18* (*nmt1:CDC6Ha cdc18::ura4*$^+$)(1), of an heterozygous diploid (*cdc18*$^+$/*cdc18::ura4*$^+$) (2), and of a wild type diploid (*cdc18*$^+$/*cdc18*$^+$) (3). The blot was hybridized with the *Eco*RV genomic wild-type clone of *cdc18*$^+$. Also shown (B) are restriction maps of genomic *cdc18*$^+$ and *cdc18*::*ura4*$^+$, the latter including an extra *Eco*RV restriction site (indicated as RV) carried by the *ura4*$^+$ gene. (C), clones isolated from the *nmt1*:*CDC6Ha cdc18*::*ura4*$^+$ strain tested above (upper 4) or the 972h$^-$ wild-type (lower 4) were streaked out onto plates lacking thiamine and replica-plated onto Petri dishes lacking or having the vitamin to show that the expression of *CDC6* supported cell growth. (D), immunoblot detection of Cdc6 protein in *S. pombe* cells. Protein extracts prepared from *nmt1*:*CDC6Ha cdc18*::*ura4*$^+$ cells repressed for 4 hours in thiamine-containing medium (1), or from *nmt1*:*CDC6Ha cdc18*::*ura4*$^+$ cells growing in medium lacking thiamine (2), or *nmt1*:*CDC6Ha* cells transformed with pDW232*ntf1*$^+$ and induced for 16 (3) or 24 hours (4) were analysed by immunoblotting with 12CA5 α-Ha monoclonal antibody to detect Cdc6Ha or rabbit antibody to Cdc2, the latter as a loading control. An extract from 972h$^-$ wild-type cells is shown for comparison.

of the assayed colonies had the correct pattern band. None of the *nmt1:CDC6HA(sup3-5) cdc18*::*ura4*$^+$ *S. pombe* clones were able to grow in medium with thiamine, which are repressing conditions for the *nmt1*$^+$ promoter (Fig. 2C,D). Consistent with previous observations regarding the deletion of *cdc18* in fission yeast (Kelly et al., 1993), *nmt1:CDC6HA(sup3-5) cdc18*::*ura4*$^+$ *S. pombe* cells did undergo mitotic catastrophe when *CDC6* expression was repressed (data not shown). Furthermore, this strain was crossed with a *leu1-32 ura4-d18 ade6-704* to segregate *nmt1:CDC6HA(sup3-5)* from *cdc18::ura4*$^+$. After sporulation, the behaviour of the haploid progeny was tested by random spore analysis. Although *nmt1:CDC6HA(sup3-5)* single mutants were isolated, no *cdc18::ura4*$^+$ single mutants (uracile prototrophs) were obtained from this cross, ruling out the possibility of second-site supressors of the deletion of the *cdc18* gene. Together, these results indicate that *CDC6* needs to be over-expressed to rescue the lack of *cdc18*. It is worth noting

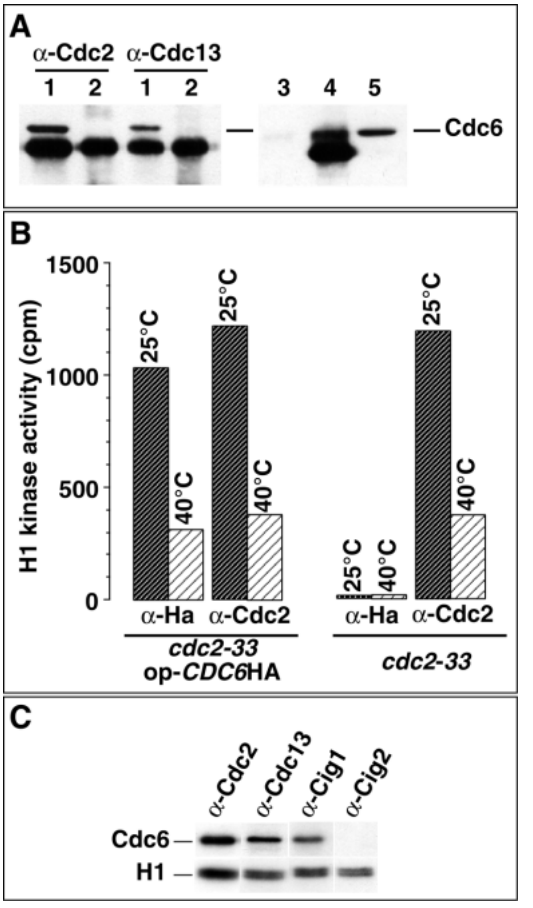

Fig. 3. Cdc6 interacts in vivo with Cdc2 kinase. (A) Cdc6 coimmunoprecipitates with Cdc2 or Cdc13. Equal amounts of protein extracts from *nmt1:CDC6HA* integrants were incubated with α-Cdc2, α-Cdc13 (as indicated), electrophoresed and analysed by immunoblotting with 12CA5 antiserum. Lanes 1, 3, 4 and 5: extracts from cells grown for 16 hours on minimal medium without thiamine. Lane 2: extracts from cells grown with thiamine. Lane 3: control sample without antibody. Lane 4: 12CA5 immunoprecipitate. Lane 5: 40 μg of total protein extract. (B) Cdc6Ha immunoprecipitation from *nmt1*-over expressing cells coprecipitates Cdc2-kinase activity. Cdc2 or hemaglutinin epitope-tagged Cdc6 were immunoprecipitated from extracts of *cdc2*-33 or *cdc2*-33 *nmt1:CDC6HA* strains grown at 25°C in minimal medium without thiamine for 16 hours. Immunoprecipitates were assayed for their ability to phosphorylate histone H1 at 25° or 40°C in vitro, and after electrophoresis were quantitated using a phosphorimager and plotted. (C) Bacterially-produced Cdc6 protein is an in vitro substrate for Cdc2. Cdc6 was expressed in *E. coli*, purified to homogeneity and used as an exogenous substrate for Cdc2, Cdc13, Cig1 and Cig2. Kinases were immunoprecipitated from *S. pombe* 972h$^-$ protein extracts with specific antibodies and assayed for their ability to phosphorylate either Cdc6 or histone H1, as indicated.

that this strain tends to diploidize frequently (up to 31% of the cfu inoculated onto phloxine plates). These results show that the Cdc6 protein can functionally substitute its putative homologue Cdc18 in *S. pombe* cells.

In vivo interaction of fission yeast Cdc2 with budding yeast Cdc6

One of the possibilities suggested by the experiments described above in *S. cerevisiae* is that $p57^{CDC6}$ is an in vivo central target for CDK in promoting the S phase, acting not only as a substrate but also recruiting Cdc28 kinase activity to the pre-replicative complex (Piatti et al., 1995; Elsasser et al., 1996). Recent biochemical evidence has indicated that Cdc18 interacts in vivo with $p34^{cdc2}$ and Orp2, a putative component of the *S. pombe* ORC (Leatherwood et al., 1996). Here we show that Cdc18 can be replaced by Cdc6, and the issue then arises of whether the budding yeast protein maintains this interaction with Cdc2. To test in vivo interactions between Cdc2 and Cdc6, we constructed two *S. pombe* strains carrying a single copy of *nmt1:CDC6HA* (see experimental procedures) in a $cdc2^+$ wild-type or in a *cdc2-33* ts mutant background. Cdc6 co-precipitated with Cdc2 and Cdc13/Cdc2 complexes in *nmt1:CDC6HA* fission yeast lysates (Fig. 3A). Cdc6 also co-precipitated with Cdc2 in a *cdc18-K46* strain background (data not shown). Moreover, Cdc6 co-immunoprecipitated a temperature-sensitive kinase activity from *cdc2-33 nmt1:CDC6HA* protein extracts (Fig. 3B). We were also interested in testing in vivo interactions between Cdc6 and Cig1/Cdc2 or Cig2/Cdc2 kinases, the latter involved in regulating G_1 progression in the mitotic cell cycle of fission yeast (Martín-Castellanos et al., 1996) by promoting the onset of the S phase (Fisher and Nurse, 1996; Mondesert et al., 1996). As shown in Fig. 4, Cdc6 co-precipitated with Cig1/Cdc2 or Cig2/Cdc2 complexes from *CDC6HA*-expressing cells. Furthermore, Cdc6 interacted in vivo with Cig1/Cdc2 and Cig2/Cdc2 through its amino moiety because a mutant Cdc6 protein, named $Cdc6^{264}$, consisting of 264 amino acids of the amino terminus co-immunoprecipitated with these kinases (Fig. 4). These data suggest that Cdc6 associates with Cdc2 in vivo. This interaction may be important to replace the Cdc18 function.

Cdc6 is an in vitro substrate for B cyclin/Cdc2 kinases

We tested whether Cdc6p could be an in vitro substrate for different *S. pombe* $p34^{cdc2}$ complexes. The *CDC6* gene was expressed in *E.coli* and the encoded protein product purified to homogeneity as judged by Coomassie Blue staining of denaturing acrylamide gels (data not shown). Kinase assays were performed immunoprecipitating Cdc2, or Cdc13, or Cig1, or Cig2 from wild-type protein extracts. Full-length Cdc6p was added to these different immunoprecipitates, using histone H1 as a standard control (Fig. 3C). Recombinant $p57^{CDC6}$ was efficiently phosphorylated by Cdc2, Cdc13, and Cig1 kinases. Nevertheless, although histone H1 control was phosphorylated, Cig2 immunoprecipitates were unable to phosphorylate the Cdc6 protein (Fig. 3C). These results indicate that $p57^{CDC6}$ is an in vitro substrate for Cdc13/Cdc2-and Cig1/Cdc2-, but not for Cig2/Cdc2-associated kinases. Since it has been shown that GST-Cdc18 is an in vitro substrate for Cig2Ha/Cdc2 kinase (Brown et al., 1997), our results regarding Cdc6 in vitro phosphorylation suggest that regulation of the *S. cerevisiae* protein when expressed in *S. pombe* may be different from controls regulating the endogenous Cdc18 protein.

$p57^{CDC6}$ overexpression induces DNA synthesis in *S. pombe*

We were interested in investigating whether *CDC6* was able to induce continuous DNA synthesis similarly to the phenotype described for $cdc18^+$ in fission yeast. Although $p57^{CDC6}$ shares significant homology with $p65^{cdc18}$, to our knowledge no data have yet been presented showing that the two genes are functionally equivalent. We reasoned that if they indeed perform the same function in their respective species it should be possible to induce continuous DNA replication by

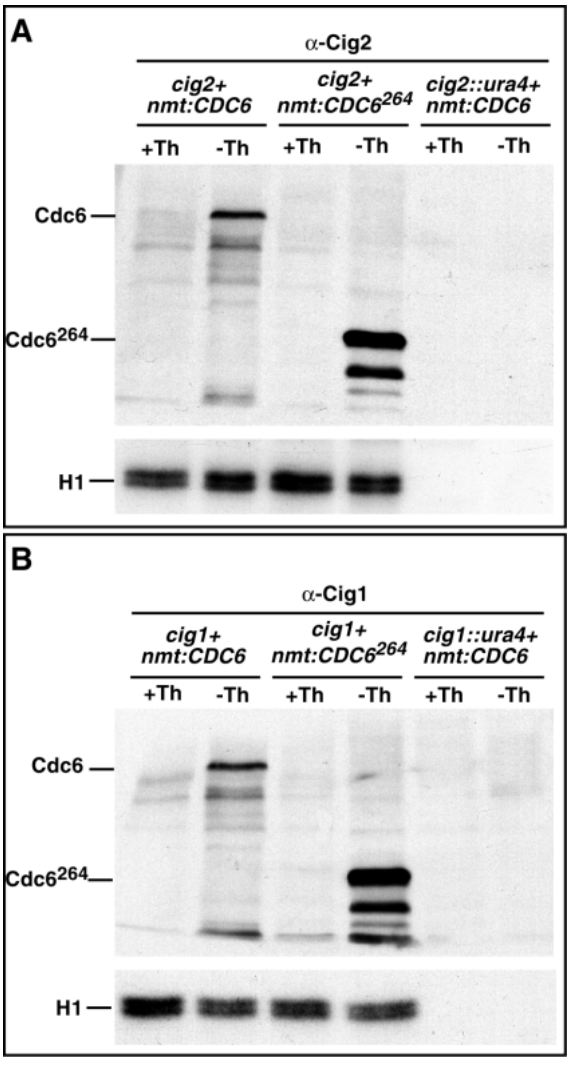

Fig. 4. Cdc6 interacts in vivo with Cig1/Cdc2 and Cig2/Cdc2 kinases. Equal amounts of protein extracts from *nmt1:CDC6HA*, *nmt1:CDC6*^{264}*HA*, *cig1*::*ura4*$^+$ *nmt1:CDC6HA* or *cig2:: ura4*$^+$ *nmt1:CDC6HA* integrants were incubated with α-Cig2, α-Cig1 (A or B, as indicated), electrophoresed and analysed by immunoblotting with 12CA5 antiserum. Protein extracts were obtained from cells grown for 16 hours on minimal medium with thiamine (+Th) or without thiamine (-Th). Negative controls were protein extracts from cells deleted for *cig2* (A) or *cig1* (B). Aliquots of the immunoprecipitates were assayed for their ability to phosphorylate histone H1 as shown below immunoblots.

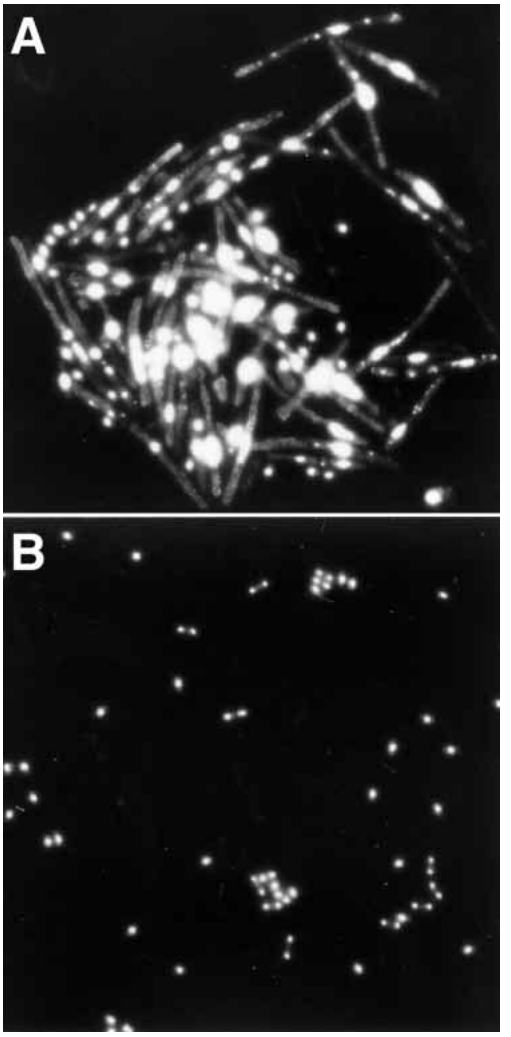

Fig. 5. *nmt1* driven *CDC6* expression induces continuous DNA synthesis in *S. pombe* cells. (A) DAPI staining of wild-type *S. pombe* cells expressing *CDC6* after 24 hours of induction on minimal medium without thiamine compared to (B) a control not expressing *CDC6*. In both cases, cells carried extra copies of *ntf1*$^+$ on an episomal vector. Note the single enlarged nucleus in *CDC6*-expressing cells compared with the wild-type control cells.

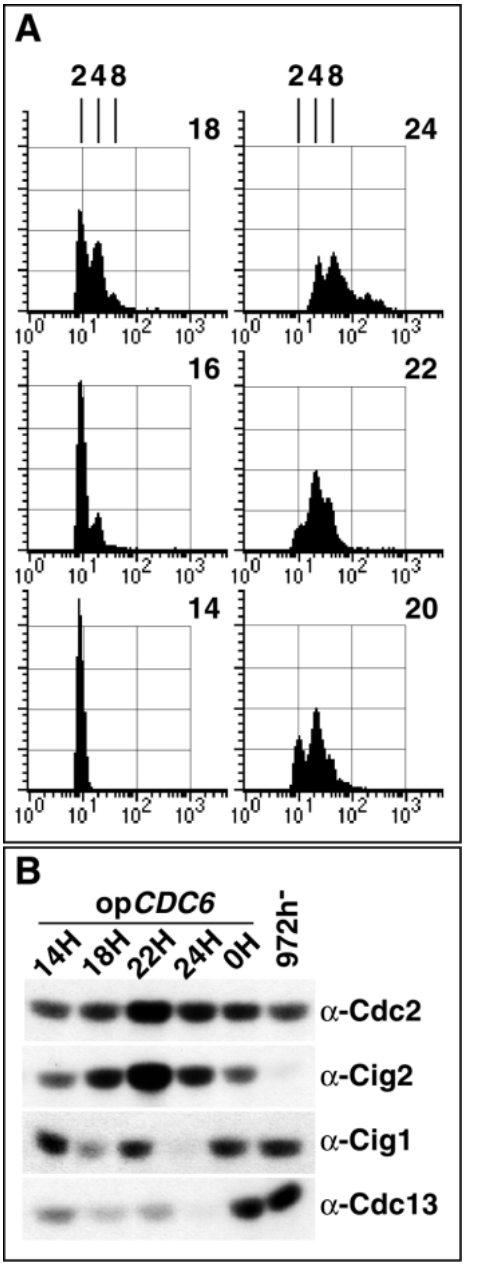

Fig. 6. Cig2-associated Cdc2 kinase predominates on Cdc6 dependent over-replicating *S. pombe* cells. (A) DNA content analysis of *S. pombe* cells massively overexpressing *CDC6* as in Fig. 5. Following inoculation on minimal medium lacking thiamine, aliquots were collected at 14, 16, 18, 20, 22 and 24 hours and divided into two for further analysis. Flow cytometry was performed on half of the cells previously fixed with ethanol. (B) Histone H1 kinase assays were performed with Cdc2, or Cdc13, or Cig1, or Cig2 kinases immunoprecipitated from total protein extracts. The samples described above were divided into four and incubated with α-Cdc2, α-Cdc13, α-Cig1, α-Cig2 antibodies, respectively. Phosphorylated histone was resolved by SDS-PAGE and visualised by autoradiography.

expressing *CDC6* in fission yeast. *adh*-driven *CDC6* overexpression causes a mitotic delay in *S. pombe* (Bueno and Russell, 1992) but does not induce over-replication. In an attempt to achieve higher levels of *CDC6* expression, we took the advantage of the *nmt1* promoter to construct a plasmid in which we placed the *CDC6* ORF under the control of the strongest *nmt1* promoter (Forsburg, 1993), first reported by Maundrel to be strongly repressed by thiamine (Maundrell, 1989). Upon the removal of thiamine this promoter is slowly induced, maximum expression being achieved after 12 to 14 hours of incubation in medium lacking this vitamin. Consistent with a delay in G_2, integration of a *nmt1*-controlled copy of the gene rendered elongated but viable populations of *S. pombe* cells when grown on medium without thiamine. To further increase the level of *CDC6*-induced expression, we transformed *nmt1:CDC6* fission yeast with a multicopy vector carrying *ntf1*$^+$, a transcription factor involved in the regulation of *nmt1*$^+$ expression (Tang et al., 1994). Incubation in medium without thiamine strongly enhanced the expression of *CDC6*, as demonstrated by western blot (Fig. 2D). Under these circumstances, *nmt1*-driven $p57^{CDC6}$ expression caused

successive rounds of DNA synthesis in the absence of mitosis or cell division in *S. pombe* cells (Figs 5 and 6), with the distinctive phenotype already described for *cdc18*$^+$ overexpressing fission yeast cells (Nishitani and Nurse, 1995; Muzi-Falconi et al., 1996). Given that the *CDC6*-dependent over-replication phenotype observed in the fission yeast could be an indirect effect of *CDC6* gene expression (e.g. interfering with cell cycle progression could result in Cdc18 stabilisation), we repeated this experiment in a *cdc18* mutant strain background and in a *cdc18* deletion strain carrying a *nmt1:CDC6HA* (Fig. 7). Under appropriate conditions, *cdc18-K46 nmt1:CDC6* cells transformed with multicopy *ntf1*$^+$ also underwent multiple rounds of DNA replication. Cells deleted for *cdc18* also re-replicated under similar conditions, indicating that *CDC6HA*-induced DNA replication is independent of the presence of *cdc18*. Our data suggest that a functional Cdc6 is able to promote continuous DNA synthesis in *S. pombe* yeast cells.

DNA synthesis requires Cig2/Cdc2 kinase activity

Studies on fission yeast have suggested that Cig2/Cdc2 is likely to be a major G_1 cyclin that normally promotes the onset of the S phase in *S. pombe* (Martín-Castellanos et al., 1996; Mondesert et al., 1996). It has also been described that in the absence of G_1 counterparts a single cyclin-associated kinase activity can promote both the S phase and mitosis in fission yeast (Fisher and Nurse, 1996; Mondesert et al., 1996). We were interested in understanding the mechanism underlying the Cdc6-associated *S. pombe* over-replication phenotype, and, in particular, determining which cyclin kinase complex promoted successive rounds of DNA replication. Upon Cdc6 induction in a multicopy *ntf1*$^+$ background, samples were taken at regular intervals and divided into two groups for further analysis. DNA content was monitored by flow cytometry in one of the aliquots (Fig. 6A), and protein extracts were prepared from the other. To test the ability of different cyclin complexes to phosphorylate histone H1 in vitro, we assayed kinase activity in Cdc2, Cdc13, Cig1 and Cig2 immunoprecipitates obtained from cell lysates (Fig. 6B). While Cdc13/Cdc2-and Cig1/Cdc2-associated kinase activity fell significantly during the time course, Cig2/Cdc2 activity remained high, accounting for most if not all Cdc2 phosphorylating capability.

If the Cdc6-dependent over-replicating phenotype relies exclusively on Cig2/Cdc2, it should be speculated that Cdc6 overexpressing cells deleted for *cig2* would not undergo continuous DNA synthesis. Therefore, we constructed two different *S. pombe* strains carrying *nmt1:CDC6* and multicopy *ntf1*$^+$, one deleted for *cig1*$^+$ (as a negative control) and the other deleted for *cig2*$^+$ (see Materials and Methods). As expected, upon induction of Cdc6 this over-replication phenotype was abolished in the *cig2* deletion strain whereas *cig1*-deleted cells underwent successive rounds of the S phase (Fig. 8). Together, these results suggest that the kinase activity of the Cig2/Cdc2 complex promotes the S-phase in Cdc6-expressing fission yeast cells.

Our results suggesting the dependence of Cdc6-induced continuous DNA synthesis on Cig2/Cdc2 are inconsistent with the published data regarding the inhibitory regulation of Cdc18 by Cig2/Cdc2 (Jallepalli et al., 1997; López-Girona et al., 1998). These two groups have shown that Cig2 antagonizes *cdc18*$^+$ function in vivo because the *cdc18-K49* mutation is supressed by deletion of cig2. We therefore examined the ability of *cdc18*$^+$ overexpression to induce DNA re-replication in *cig2*$^+$ wild-type or *cig2* mutant backgrounds. We constructed two strains: *cig2*$^+$ *nmt1:cdc18*$^+$ and *cig2::LEU2 nmt:cdc18*$^+$. Deletion of *cig2* enhanced the phenotype of *nmt1:cdc18*$^+$ cells under repressing conditions, resulting in an elongated cell length phenotype, dividing ~10% longer than the wild-type (15.5 μm versus 14 μm). Nevertheless, upon removal of thiamine *cig2* mutant cells re-replicated with kinetics similar to that of the *cig2*$^+$ controls (data not shown), indicating that Cdc18-induced re-replication does not depend entirely on Cig2/Cdc2 activity, unlike Cdc6 re-replication. These data suggest that some important aspects of the continuous DNA synthesis phenotype driven by Cdc18 and Cdc6 differ significantly.

Cdc6-dependent DNA over-replication in fission yeast does not require continuing protein synthesis

It has been shown that *S. pombe* cells containing high levels of Cdc18 do undergo continuous DNA replication in the absence of protein synthesis (Nishitani and Nurse, 1995). Therefore, we studied whether *CDC6*-expressing *S. pombe* cells, containing high levels of the Cdc6 protein, continue to undergo successive rounds of DNA replication even when further protein synthesis was inhibited by cycloheximide. Upon removal of thiamine,

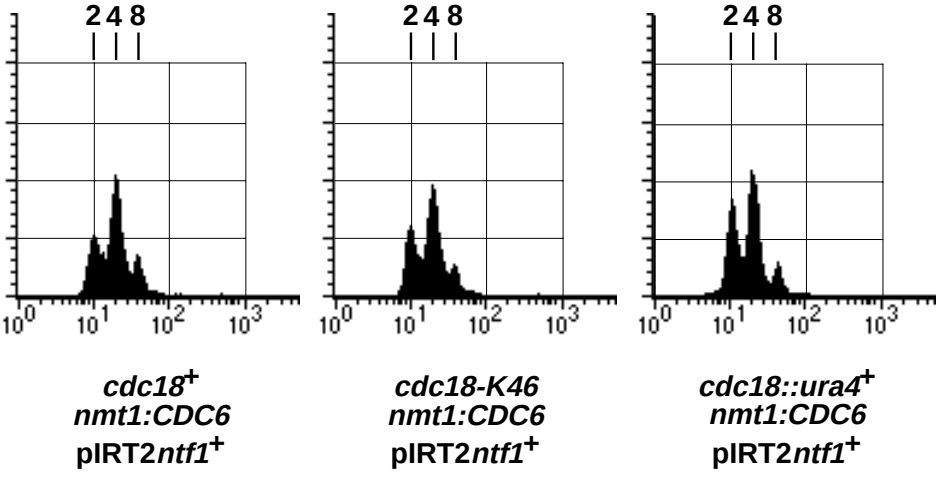

Fig. 7. *CDC6*-induced DNA over-replication is independent of the presence of *cdc18*$^+$. DNA content analysis of wild-type *cdc18*$^+$, *cdc18-K46* and *cdc18*::*ura4*$^+$ deletion *S. pombe* strains, after 20 hours of induction on minimal medium without thiamine at 35.5°C. In all cases, cells carried extra copies of *ntf1*$^+$ on an episomal vector (pIRT2, a *LEU2*/*ars1 S. pombe* plasmid).

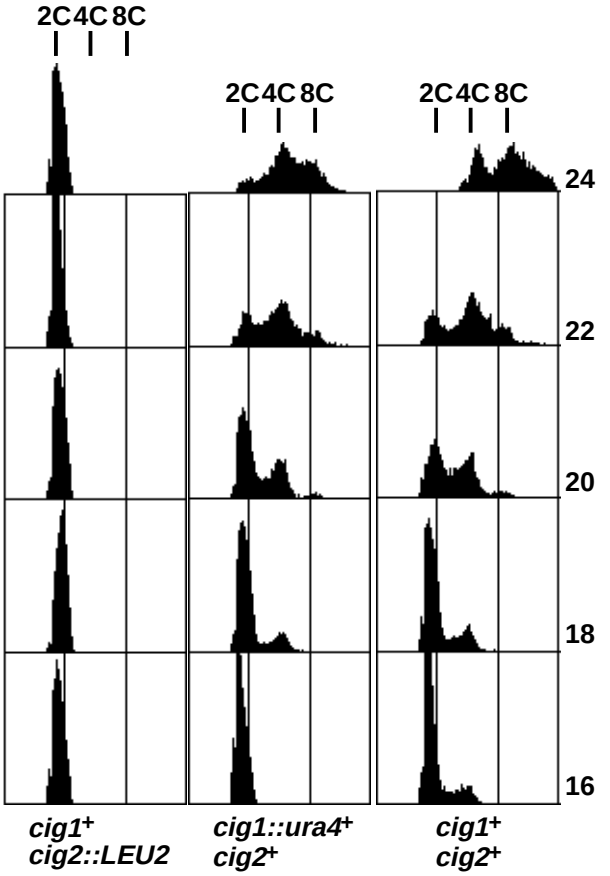

Fig. 8. *S. pombe* cells lacking *cig2* do not undergo Cdc6-dependent over-replication. DNA content analysis of *S. pombe* cells deleted for *cig2*, *cig1*, or wild-type for both B-type cyclin genes and massively overexpressing *CDC6* as in figures 5 and 6. After inoculation on minimal medium lacking thiamine, samples were collected at 16, 18, 20, 22, and 24 hours (as indicated). In all cases, cells carried extra copies of *ntf1*$^+$ on an episomal vector. Flow cytometry was performed on cells previouly fixed with ethanol. Although *cig1*$^+$ *cig2*$^+$ wild-type and *cig1::ura4*$^+$ *cig2*$^+$ cells over-replicated their DNA, *cig2* deleted cells did not undergo re-replication.

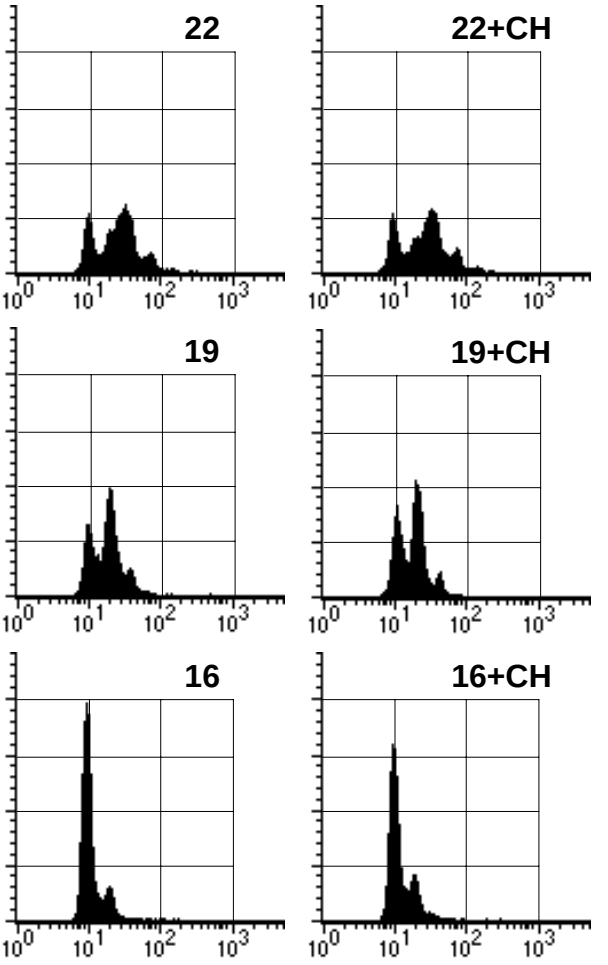

Fig. 9. DNA over-replication does not require continuing protein synthesis. A *nmt1:CDC6* integrant derepressed at time 0 was incubated in minimal medium lacking thiamine for 16 hours at 32°C and divided into two aliquots. After the addition of cycloheximide (CH) to one of the samples, cells were collected at 3 hour intervals and fixed with ethanol. DNA contents were measured by flow cytometry. Plots of control (left panels) and cycloheximide-treated cells (right panels) are shown.

CDC6 was overexpressed for 16 hours at 32°C. The culture was split in two and cycloheximide was added to one half. The DNA contents of cells were determined by flow cytometry from samples taken three and six hours after addition of the drug (Fig. 9). The DNA content continued to increase even though protein synthesis was inhibited.

DISCUSSION

Several lines of evidence presented here suggest that proteins encoded by *S. cerevisiae CDC6* and *S. pombe cdc18*$^+$ genes are functional homologues. This conclusion is largely based on the rescue of both the thermosensitive cell cycle arrest of the *cdc18*-K46 allele and the lethal phenotype of the deletion of *cdc18* by the expression of *CDC6* under the control of the *nmt1*$^+$ promoter in fission yeast cells (Figs 1 and 2). Although the suppression of the *cdc18*-K46 by *CDC6* suggests that both genes may play the same role, it should be mentioned that the allele *cdc18*-K46 itself in a multicopy vector is able to rescue *cdc18* ts mutant strains (Kelly et al., 1993), indicating that this protein is partially active at the restrictive temperature. In formal terms, therefore, it is possible that the expression of *CDC6* may indirectly cause *cdc18*-K46 to be accumulated, resulting in complementation of the *ts* defect. We therefore undertook to determine whether *S. cerevisiae CDC6* was functionally equivalent to *cdc18*$^+$. *S. pombe* cells deleted for *cdc18*, a putative *CDC6* homologue sharing 28% identity along the amino acid sequence, are defective in DNA replication but lethally divide (Kelly et al., 1993). *S. cerevisiae* cells devoid of a functional Cdc6 also show a similar dual defect (Piatti et al., 1995). Despite this degree of homology and considering that they perform the same role in S phase initiation, it has been reported that these proteins are unable to replace each other (Dutta and Bell, 1997). By deleting the endogenous *cdc18*$^+$ gene in a fission yeast integrant carrying a copy of *nmt1:CDC6* we show that *cdc18* is replaceable by *CDC6* (Fig. 2). Our data

are consistent with the rescue of both the absence of DNA replication and the lethal cell division that cells deleted for the initiator Cdc18 protein undergo. Nevertheless, spores deleted for *cdc18* carrying *nmt1:CDC6* were unable to germinate (unpublished data). A possible explanation for this could be that *CDC6* may be only partially able to rescue *cdc18*. To understand whether or not *CDC6* fully complements $cdc18^+$, experiments are currently underway to replace $cdc18^+$ by a single copy of the *CDC6* ORF controlled by the endogenous wild-type $cdc18^+$ promoter.

If Cdc6 functionally replaces Cdc18, it is reasonable to assume that the *S. cerevisiae* protein would retain the molecular interactions of its homologue. It has been described that in vivo Cdc18 interacts with Cdc2 through its amino terminus (Leatherwood et al., 1996; Brown et al., 1997; López-Girona et al., 1998). Indeed, we observed that Cdc6 co-immunoprecipitated Cdc2-associated kinase activity and that Cdc6Ha was present in immunoprecipitates of Cdc2, Cdc13, Cig1, and Cig2 (Figs 3A,B and 4), demonstrating that the initiator protein interacts in vivo with the CDK. In fact, Cdc6 interacts in vivo with Cdc2 in *S. pombe* cells through its amino moiety (as shown for Cig1 and Cig2, Fig. 4) because a non-functional mutant Cdc6 protein, $Cdc6^{264}$, consisting of 264 amino acids of the amino terminus co-immunoprecipitated Cdc13 and Cdc2-associated kinase, while the carboxy terminus moiety did not (unpublished data). Furthermore, we observed that recombinant Cdc6 protein purified from *E. coli* was a substrate for Cdc2, Cdc13 and Cig1 kinases but, perhaps significantly, it was not an in vitro substrate for the Cig2-associated Cdc2 (Fig. 3C). Again, this is comparable with the findings for the Cdc18 protein because it has been shown that this protein is an in vitro substrate for the Cdc2 kinase (Brown et al., 1995; Jallepalli et al., 1997; López-Girona et al., 1998). Nevertheless, it has been shown that Cdc18 is an in vitro substrate for the Cig2 kinase (Brown et al., 1997), suggesting that controls regulating Cdc6 in *S. pombe* differ from those acting on the endogenous Cdc18. Alternatively, our results could also suggest that although the Cdc6/Cdc18 class of initiator proteins may interact in vivo with Cig2/Cdc2 they may be poor substrates for this kinase. If this is the case, recruiting Cig2-associated Cdc2 activity would be a major role for the initiator protein for the kinase to induce DNA replication. This model is supported by the strong physical interaction between Cdc18 or Cdc6 with Cig2/Cdc2 (López-Girona et al., 1998; Fig. 4) and consistent with the notion of Cdc13/Cdc2 being the major inhibitory kinase of Cdc18 in vivo, as has been suggested (López-Girona et al., 1998).

Comparably to the role of the Cdc18 initiator protein in fission yeast (Nishitani and Nurse, 1995; Muzi-Falconi et al., 1996), we have also shown in this work that massive overexpression of $p57^{CDC6}$ in *S. pombe* is able to induce successive rounds of DNA replication in the absence of mitosis, resulting in elongated cells with huge nuclei (Fig. 5). Actually, $p57^{CDC6}$ induces continuous DNA replication in a $cdc18^+$ wild-type, in a *cdc18*-K46 mutant strain, or in a *cdc18* deletion strain background (Fig. 7), suggesting that this phenotype relies on a functional Cdc6 protein. Consistent with this idea, a non-functional $cdc6^{264}$ is unable to promote over-replication (unpublished data).

By measuring kinase activity in replicating cells our experiments suggest that Cig2/Cdc2 accounts for most if not all the phosphorylating activity present in cells over-replicating their DNA (Fig. 6) whereas Cdc13/Cdc2 and Cig1/Cdc2 complexes are strongly inhibited. Consistent with the kinase assays, the deletion of *cig2* blocks the CDC6-dependent DNA over-replication phenotype (Fig. 8). These two lines of evidence suggest that Cdc6 is able to induce DNA replication in fission yeast in a Cig2/Cdc2-dependent manner, the same kinase being unable to phosphorylate the Cdc6 protein in vitro. Despite this, it has been described that the Cig2 kinase counteracts the positive effect of Cdc18 because deletion of the cyclin gene rescues the *cdc18*-K46 mutation (Jallepalli et al., 1997) and it has been shown that Cdc18 interacts in vivo with Cig2 (López-Girona et al., 1998). It is possible that Cdc6 in *S. pombe* would not be inhibited by the Cig2 kinase due to its heterologous origin. Consistent with this idea, the re-replication induced by over-expression of $cdc18^+$ does not depend on the presence of a functional $cig2^+$ B-type cyclin gene (data not shown), suggesting that the *S. cerevisiae* Cdc6 protein is missing one of the controls acting on Cdc18, namely Cig2-mediated inhibitory phosphorylation.

It is intriguing that over-expression of the *CDC6* gene causes continuous DNA synthesis in *S. pombe* cells while vast overexpression of the same gene in *S. cerevisiae*, the natural host of the gene, does not drive cells into re-replication, at least in some strain backgrounds (Drury et al., 1997). Although both fission and budding yeast might ensure one replication per cell cycle by inhibiting the re-formation of pre-replicative complexes once replication has been initiated at the origins (Dutta and Bell, 1997), perhaps the major control that the fission yeast has is CDK-mediated regulation of Cdc18 proteolysis (Jallepalli et al., 1997; López-Girona et al., 1998), while the budding yeast would block re-replication by CDK-dependent phosphorylation of different substrates by regulating both the localisation of some of them and the proteolysis of others (Elsasser et al., 1996; Drury et al., 1997; Liang and Stillman, 1997; Sánchez et al., 1999). Such circumstances would explain why the over-expression of *CDC6* drives *S. pombe* cells into successive rounds of DNA synthesis, despite its inability to do so in *S. cerevisiae*.

We thank Francisco Antequera, Jaime Correa, Karim Labib, Sergio Moreno, and the ASP lab for many helpful discussions. We thank Karim Labib for critically reading this manuscript. We also thank N. Skinner for correcting the English text. This research was supported by CICYT and Fundación Ramón Areces grants to A.B.

REFERENCES

Aparicio, O. M., Weinstein, D. M. and Bell, S. P. (1997). Components and dynamics of DNA replication complexes in *S. cerevisiae*: redistribution of Mcm proteins and Cdc45p during S phase. *Cell* **91**, 59-69.

Basco, R. D., Segal, M. D. and Reed, S. I. (1995). Negative regulation of G_1 and G_2 by S-phase cyclins of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **15**, 5030-5042.

Bell, S. P. and Stillman, B. (1992). Nucleotide-dependent recognition of chromosomal origins of DNA replication by a multi-protein complex. *Nature* **357**, 128-134.

Brown, G. W., Jallepalli, P. V., Huneycutt, B. J. and Kelly, T. J. (1997). Interaction of the S phase regulator Cdc18 with cyclin-dependent kinase in fission yeast. *Proc. Nat. Acad. Sci. USA* **94**, 6142-6147.

Bueno, A. and Russell, P. (1992). Dual functions of *CDC6*: a yeast protein required for DNA replication also inhibits nuclear division. *EMBO J.* **11**, 2167-2176.

Cocker, J. H., Piatti, S., Santocanale, C., Nasmyth, K. and Diffley, J. F. X. (1996). An essential role for the Cdc6p protein in budding yeast pre-replicative complexes. *Nature* **379**, 180-182.

Coleman, T. R., Carpenter, P. B. and Dunphy, W. G. (1996). The Xenopus Cdc6 protein is essential for the initiation of a single round of DNA replication in cell-free extracts. *Cell* **87**, 53-63.

Diffley, J. F. K. and Cocker, J. H. (1992). Protein-DNA interactions at a yeast replication origin. *Nature* **357**, 169-172.

Diffley, J. F. X., Cocker, J. H., Dowell, S. J. and Rowley, A. (1994). Two steps in the assembly of complexes at yeast replication origins in vivo. *Cell* **78**, 303-316.

Donovan, S., Harwood, J., Drury, L. S. and Diffley, J. F. X. (1997). Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. *Proc. Nat. Acad. Sci. USA* **94**, 5611-5616.

Dowell, S. J., Romanowski, P. and Diffley, J. F. X. (1994). Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins in vivo. *Science* **265**, 1243-1246.

Drury, L. S., Perkins, G. and Diffley, J. F. X. (1997). The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast. *EMBO J.* **16**, 5966-5976.

Dutta, A. and Bell, S. P. (1997). Initiation of DNA replication in eukaryotic cells. *Annu. Rev. Cell Dev. Biol.* **13**, 293-332.

Elsasser, S., Lou, F., Wang, B., Campbell, J. L. and Jong, A. (1996). Interaction between yeast Cdc6 protein and B-type cyclin/Cdc28 kinases. *Mol. Biol. Cell.* **7**, 1723-1735.

Fisher, D. L. and Nurse, P. (1996). A single fission yeast mitotic cyclin B $p34^{cdc2}$ kinase promotes both S-phase and mitosis in the absence of G_1 cyclins. *EMBO J.* **15**, 850-860.

Forsburg, S. L. (1993). Comparison of *Schizosaccharomyces pombe* expression systems. *Nucl. Acids Res.* **21**, 2955-2956.

Guthrie, C. and Fink, G. R. (1991). Guide to yeast genetics and molecular biology. *Meth. Enzymol.* **194**, 1-933.

Hateboer, G., Wobst, A., Petersen, B. O., Le Cam, L., Vigo, E., Sardet, C. and Helin, K. (1998). Cell-cycle regulated expression of mammalian CDC6 is dependent on E2F. *Mol. Cell. Biol.* **18**, 6679-6697.

Hartwell, L. H. (1976). Sequential function of gene products relative to DNA synthesis in the yeast cell cycle. *J. Mol. Biol.* **104**, 803-817.

Hogan, E. and Koshland, D. (1992). Addition of extra origins of replication to a minichromosome suppressed its mitotic loss in *cdc6* and *cdc14* mutants of *Saccharomyces cerevisiae*. *Proc. Nat. Acad. Sci. USA* **89**, 3098-3102.

Hutter, K. J. and Eipel, H. E. (1979). Microbial determinations by flow cytometry. *J. Gen. Microbiol.* **113**, 369-375.

Jallepalli, P. V., Brown, G. W., Muzi-Falconi, M., Tien, D. and Kelly, T. J. (1997). Regulation of the replication initiator protein $p65^{cdc18}$ by CDK phosphorylation. *Genes Dev.* **11**, 2767-2779.

Kelly, T. J., Martin, G. S., Forsburg, S. L., Stephen, R. J., Russo, A. and Nurse, P. (1993). The fission yeast *cdc18*$^+$ gene product couples S-phase to start and mitosis. *Cell* **74**, 371-382.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Leatherwood, J., López-Girona, A. and Russell, P. (1996). Interaction of Cdc2 and Cdc18 with a fission yeast ORC-like protein. *Nature* **379**, 360-363.

Liang, C., Weinreich, M. and Stillman,B. (1995). ORC and Cdc6p interact and determine the frequency of initiation of DNA replication in the genome. *Cell* **81**, 667-676.

Liang, C. and Stillman, B. (1997). Persistent initiation and chromatin-bound MCM proteins during the cell cycle in *cdc6* mutants. *Genes Dev.* **11**, 3375-3386.

López-Girona, A., Mondesert, O., Leatherwood, J. and Russell, P. (1998). Negative regulation of Cdc18 DNA replication protein by Cdc2. *Mol. Biol. Cell* **9**, 63-73.

Martín-Castellanos, C., Labib, K. and Moreno, S. (1996). B-type cyclins regulate G_1 progression in fission yeast in opposition to the $p25^{rum1}$ CDK inhibitor. *EMBO J.* **15**, 839-849.

Maundrell, K. (1989). *nmt1* of fission yeast: a highly transcribed gene completely repressed by thiamine. *J. Biol. Chem.* **265**, 10875-10864.

Millar, J., McGowan, C., Lenaers, G., Jones, R. and Russell, P. (1991). $p80^{cdc25}$ mitotic inducer is the tyrosine phosphatase that activates $p34^{cdc2}$ kinase in fission yeast. *EMBO J.* **10**, 4301-4309.

Mondesert, O., McGowan, C. H. and Russell, P. (1996). Cig2, a B-type cyclin promotes the onset of S-phase in *Schizosaccharomyces pombe*. *Mol. Cell Biol.* **16**, 1527-1533.

Moreno, S., Klar, A. and Nurse, P. (1991). Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Meth. Enzymol.* **194**, 795-823.

Muzi-Falconi, M., Brown, G. W. and T. Kelly. (1996). *cdc18*$^+$ regulates initiation of DNA replication in *Schizosaccharomyces pombe*. *Proc. Nat. Acad. Sci. USA* **93**, 1566-1570.

Nasmyth, K. and Nurse, P. (1981). Cell division cycle mutants altered in DNA replication and mitosis in the fission *yeast Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **182**, 119-124.

Nishitani, H. and Nurse, P. (1995). $p65^{cdc18}$ plays a major role controlling the initiation of DNA replication in fission yeast. *Cell* **83**, 397-405.

Piatti, S., Lengauer, C. and Nasmyth, K. (1995). Cdc6 is an unstable protein whose *de novo* synthesis in G_1 is important for the onset of S phase and for preventing a 'reductional' anaphase in the budding *yeast Saccharomyces cerevisiae*. *EMBO J.* **14**, 3788-3799.

Sadhu, K., Reed, S. I., Richardson, H. and Russell, P. (1990). Human homolog of fission yeast *cdc25* mitotic inducer is predominantly expressed in G_2. *Proc. Nat. Acad. Sci. USA* **87**, 5139-5143.

Sambrook, J., Fritsch, E. and Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Second edition. Cold Spring Harbor Laboratory Press.

Sánchez, M. M., Calzada, J. A. and Bueno, A. (1999). The S-phase initiator Cdc6 protein is ubiquitinated in vivo for proteolysis in *Saccharomyces cerevisiae. J. Biol. Chem.* **274**, 9092-9098.

Tanaka, T., Knapp, D. and Nasmyth, K. (1997). Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. *Cell* **90**, 649-660.

Tang, C. S. L., Bueno, A. and Russell, P. (1994). *ntf1*+ encodes a Cys6 zinc finger-containing transcription factor that regulates the *nmt1* promoter in fission yeast. *J. Biol Chem*. **269**, 11921-11926.

Williams, R. S., Shohet, R. V. and Stillman, B. (1997). A human protein related to yeast Cdc6p. *Proc. Nat. Acad. Sci. USA* **94**, 142-147.

Yan, Z., DeGregori, J., Shohet, R., Leone, G., Stilman, B., Nevins, J. R. and Williams, R. S. (1998). Cdc6 is regulated by E2F and is essential for DNA replication in mammalian cells. *Proc. Nat. Acad. Sci. USA* **95**, 3603-3608.

Zou, L., Mitchell, J. and Stillman, B. (1997). *CDC45*, a novel gene that functions with the origin recognition complex and Mcm proteins in initiation of DNA replication. *Mol. Cell. Biol.* **17**, 553-563.